# IMMUNOSTIMULANT OLIGONUCLEOTIDE

The present invention relates to the domain of immunostimulants. More particularly, the invention relates to oligonucleotides capable of stimulating human cells involved in the immune system, and to the use thereof as an immunization adjuvant.

A large number of oligonucleotides have already been described in the prior art, in relation to their immunostimulant properties. Thus, application EP0468520 describes immunostimulant polynucleotides consisting of a linear DNA single strand comprising from 10 to 100 nucleotides linked together according to a palindromic sequence.

According to application WO 96/02555, the immunostimulatory activity of oligonucleotides is linked to the presence of a 5′ CG 3′ dinucleotide sequence in which C is not methylated, the immunostimulant activity being greater if the CG unit is preceded in 5′ by the dinucleotide GA and/or followed in 3′ by the dinucleotide TC or TT.

On the other hand, according to patent application WO 98/52962, it is not necessary for the oligonucleotides to have a minimum of 8 nucleotides, as had been described previously, or for their sequence to be a palindrome, or even for them to comprise the dinucleotide CG; thus, this application describes the following 3 oligonucleotides for their use as an immunization adjuvant:
5′ GACGTT 3′, 5′ GAGCTT 3′, and 5′ TCCGGA 3′.

According to US patent 5,663,153, the immunostimulant activity of oligonucleotides is not linked to the sequence of the nucleotides, but to the nature of the bond between nucleotides, the presence of at least one

phosphorothioate bond making it possible to induce stimulation of the immune system.

Most tests of the prior art for evaluating the immunostimulant activity of the oligonucleotides provided are carried out either in vitro, on animal cells (essentially murine cells), or in vivo on mice. However, the differences which exist between the immune system of mice and that of humans have led to differences between the results obtained on murine cells and those obtained on human cells. It is therefore not certain that all the oligonucleotides which have been described as immunostimulant in the prior art really are immunostimulant with respect to humans.

Now, the pharmaceutical industry is in great need of immunostimulants which can be administered to humans, in particular in the field of vaccines.

The aim of the present invention is therefore to propose oligonucleotides capable of stimulating cells of the immune system of humans.

In order to achieve this aim, a subject of the invention is an oligonucleotide capable of stimulating human cells of the immune system, characterized in that it comprises at least one sequence 5′ T T $N_1$ $N_2$ T T 3′ in which T is thymine, and $N_1$ and $N_2$ may each represent adenine, thymine, cytosine or guanine, and in that it lacks a dinucleotide CG in which the cytosine C is not methylated.

A subject of the invention is also the use of such an oligonucleotide, for manufacturing a medicinal product.

According to one characteristic of the invention, the oligonucleotide comprises from 6 to 100 nucleotides.

According to a particular characteristic, the oligonucleotide according to the invention is characterized in that $N_1$ represents adenine and in that $N_2$ represents guanine.

According to another characteristic, the oligonucleotide according to the invention is characterized in that the 5′ T T $N_1$ $N_2$ T T 3′ unit is repeated at least once.

According to another characteristic, the oligonucleotide according to the invention is characterized in that the 5′ T T $N_1$ $N_2$ T T 3′ unit unit is repeated twice.

According to another characteristic, the oligonucleotide according to the invention is characterized in that the repeated 5′ T T $N_1$ $N_2$ T T 3′ units are separated by a nucleotide $N_3$ which, each time, may be identical or different, and which may represent A, C, T or G.

According to a particular characteristic, the oligonucleotide according to the invention is characterized in that the nucleotide $N_3$ separating the first two $TTN_1N_2TT$ units read when the sequence is in the 5′ → 3′ orientation represents cytosine.

According to another characteristic, the oligonucleotide according to the invention is characterized in that it comprises the sequence 5′ $TTAGTTCTTAGTTN_3TTAGTT$ 3′, in which A represents adenine, T represents thymine, G represents guanine and C represents cytosine, and in which $N_3$ may signify A, T, C or G.

According to another characteristic, the oligonucleotide according to the invention is capable of inducing human lymphocyte proliferation.

According to another characteristic, the oligonucleotide according to the invention is capable of increasing the expression of the activation marker CD86 and of the receptor CD25 on human B lymphocytes.

According to another characteristic, the oligonucleotide according to the invention is capable of inducing cytokine secretion.

A subject of the invention is also an immunization adjuvant, characterized in that it comprises at least one oligonucleotide which is capable of stimulating human cells of the immune system and which contains at least one sequence 5′ T T $N_1$ $N_2$ T T 3′ in which T is thymine, and $N_1$ and $N_2$ may each represent adenine, thymine, cytosine or guanine, the oligonucleotide lacking a CG dinucleotide sequence in which the cytosine C is not methylated.

A subject of the invention is also an immunization composition for human use, comprising at least one immunization antigen, characterized in that it also comprises at least one oligonucleotide which is capable of stimulating human cells of the immune system and which contains at least one sequence 5′ T T $N_1$ $N_2$ T T 3′ in which T signifies thymine, and $N_1$ and $N_2$ may each represent adenine, thymine, cytosine or guanine, the oligonucleotide lacking a CG dinucleotide sequence in which the cytosine C is not methylated.

The present invention will be more clearly understood upon reading the following description, with reference to figures 1 to 11 which illustrate the results obtained in the tests described in Examples 2 to 7.

In particular, figures 1 and 2 indicate the number of counts per minute obtained in the test of the example.

Figures 3 and 5 indicate the percentage of CD20+ cells expressing the CD25 receptor, for the oligonucleotides obtained according to Example 1.

Similarly, figures 4 and 6 indicate the percentage of CD20+ cells expressing the CD86 marker.

Figure 7 indicates the number of counts per minute obtained in the test of Example 4.

Figure 8 indicates the percentage of CD20+ cells expressing the CD25 receptor, for the oligonucleotides obtained according to Example 4. Similarly, Figure 9 indicates the percentage of CD20+ expressing the CD86 marker.

Figure 10 indicates the number of spots measured for the secretion of γ interferon by cells stimulated in the presence of the oligonucleotides having the sequences 9 to 12 described in Example 4.

Figure 11 indicates the number of spots measured for the secretion of IL10 by cells stimulated in the presence of the oligonucleotides having the sequences 9 to 12 described in Example 4.

For the purposes of the present invention, the term "oligonucleotide" is intended to mean a polynucleotide comprising at least six nucleotides. Specifically, contrary to the teaching of the article entitled *"CpG motifs in bacterial DNA trigger direct B-cell activation"*, Krieg et al., Nature 1995, it was noted that it is not necessary for the oligonucleotide to have at least 8 nucleotides. On the other hand, the upper limit of the size of the oligonucleotides is not really determined. It may, however, be noted that, the longer the oligonucleotide, the more difficult it will be to purify it during the steps of synthesis and the higher the cost price thereof. In addition, it is

probable that a very long oligonucleotide will find it more difficult to penetrate cells. Thus, for the needs of the present invention, it is considered that limiting the size of the oligonucleotide to 100 nucleotides is suitable. This oligonucleotide is preferably a single-stranded oligonucleotide; it may be an oligodeoxyribonucleotide or an oligoribonucleotide. Particularly good results have been obtained using an oligodeoxyribonucleotide. The oligonucleotides which are suitable for the purposes of the invention may be in phosphodiester form or, in order to be more stable, in the form of phosphorothioates or phosphodiester/phosphorothioate hybrids. Those preferred are phosphorothioate oligonucleotides.

The oligonucleotide according to the invention is capable of stimulating human cells of the immune system, such as B lymphocytes, T lymphocytes, monocytes and dendritic cells. This stimulation is assessed, in particular, by lymphoproliferation or by the expression of markers, such as the cytokine receptor CD25 or the activation marker CD86 on B lymphocytes. It is possible to select the oligonucleotides of interest using tests other than those provided in the present application, on the condition, however, that they are tests which evaluate the capacity for stimulating human cells and not, as in most of the documents of the prior art, tests which evaluate the capacity for stimulating murine cells. It would, in particular, be possible to test the expression of other B lymphocyte activation markers, such as the CD69 markers, or the expression of proliferation markers, such as the KI67 marker; tests relating to an increase in activation markers and maturation markers of dendritic cells may also be used. Similarly, tests for assessing an increase in production of certain cytokines may also be used.

According to one characteristic of the invention, the oligonucleotide comprises at least one nucleotide

sequence 5′ T T $N_1$ $N_2$ T T 3′ in which T signifies thymine, and $N_1$ and $N_2$ may each represent adenine, thymine, cytosine or guanine. This formula thus covers 16 possibilities. This sequence may be 5′-terminal or 3′-terminal, or be surrounded by other nucleotides. It may be unique or repeated several times identically within the same oligonucleotide. An oligonucleotide according to the invention may also comprise several different sequences each corresponding to the 5′ T T $N_1$ $N_2$ T T 3′ unit.

According to the invention, the oligonucleotide does not comprise a palindromic sequence. Despite this absence of palindromic sequence, such an oligonucleotide is capable of stimulating human cells of the immune system.

According to one characteristic, the oligonucleotide according to the invention lacks a dinucleotide CG in which the cytosine is not methylated. This exclusion also applies to the $N_1$ $N_2$ unit. The ability of the oligonucleotides of the prior art to be immunostimulant has almost always been interpreted as being linked to the presence of nonmethylated CpG units (cf. in particular the article by Krieg et al. in Nature of April 1995, mentioned above), this interpretation being coherent with the observation according to which the frequency of this dinucleotide is approximately four times greater in the genome of bacteria than in that of vertebrates. Surprisingly, it has now been found that oligonucleotides completely lacking this dinucleotide unit are, however, entirely capable of stimulating the cells of the human immune system.

According to a particular embodiment of the present invention, the $N_1N_2$ unit corresponds to the dinucleotide AG in which A signifies adenine and G signifies guanine.

According to an advantageous characteristic, the 5′ TTAGTT 3′ unit is repeated at least once in the oligonucleotide, and preferably at least twice. More preferably, the repeated units are separated by at least one nucleotide $N_3$, which represents adenine, cytosine, guanine or thymine. Within an oligonucleotide, this separating nucleotide may always be the same or may be different each time. Preferably, the nucleotide separating the first two TTAGTT units of the oligonucleotide (taking the direction for reading to be 5′→3′) consists of cytosine.

In particular, for the purpose of the present invention, those oligonucleotides in which the nucleotide sequences correspond to the formula 5′ TTAGTTCTTAGTT$N_3$TTAGTT 3′, in which $N_3$ represents A, T, C or G, are preferred.

According to a particular characteristic, the oligonucleotide according to the invention lacks or is low in nucleotide sequence capable of inhibiting the cells of the human immune system. In fact, in order to obtain an overall immunostimulant effect, if inhibitory or neutralizing units such as, for example, those described in application WO 98/52581 are present, their effect must be suppressed or decreased, through the presence of a sequence with more pronounced immunostimulant effect or through the presence of a greater number of 5′ T T $N_1$ $N_2$ T T 3′ sequences.

A subject of the present invention is also an immunization adjuvant comprising at least one immunostimulant oligonucleotide having at least one 5′ T T $N_1$ $N_2$ T T 3′ unit as mentioned above. The term "immunization adjuvant" is intended to mean a product which makes it possible to increase or to modify the response of the immune system of an organism with respect to the administration of an antigen. In

particular, it may be an increase in the humoral response or in the cellular response.

The action of an immunization adjuvant may also be, not an increase in the response which would occur in the absence of adjuvant, but a different orientation of the response produced: for example, orientation toward a cellular response rather than a humoral response, production of certain cytokines rather than others, production of certain antibody types or subtypes rather than others, stimulation of certain cells rather than others, etc.

The immunostimulant oligonucleotide of the present invention may be used as an immunization adjuvant whatever the nature of the antigen administered and whatever the number of valencies used. It may be the only adjuvant used or, on the contrary, it may be one element of an adjuvant combination.

The adjuvant action of the oligonucleotide according to the invention may be obtained either when it is combined with the antigen(s) when they are administered, i.e. when they are part of the same immunization composition, or when it is administered separately from the antigen(s). It is, however, preferred to use it in the same immunization composition as the antigen(s) to be administered.

The oligonucleotide according to the invention may advantageously be administered via all the routes which can be used for an immunization composition: mucosal route or systemic route.

One of the subjects of the invention is an immunization composition comprising at least one immunostimulant oligonucleotide having a 5′ T T $N_1N_2$ T T 3′ sequence as described above.

An immunization composition according to the invention may be intended for immunization against a single disease or intended for immunization against several diseases. It may be a liquid immunization composition or a lyophilized composition. It may comprise, besides the antigens, all or some of the components conventionally present in a vaccine, such as buffers, stabilizers, preserving agents, etc. It may also comprise one or more adjuvant(s) other than those which are subjects of the present invention. It may also comprise several adjuvants which are subjects of the present invention, consisting either of oligonucleotides which all have the same 5′ T T $N_1$ $N_2$ TT 3′ unit but which differ by the nucleotides in 5′ and/or in 3′, or nucleotides which have different 5′ T T $N_1$ $N_2$ T T 3′ units, the sequence in 5′ and in 3′ of which are identical or different.

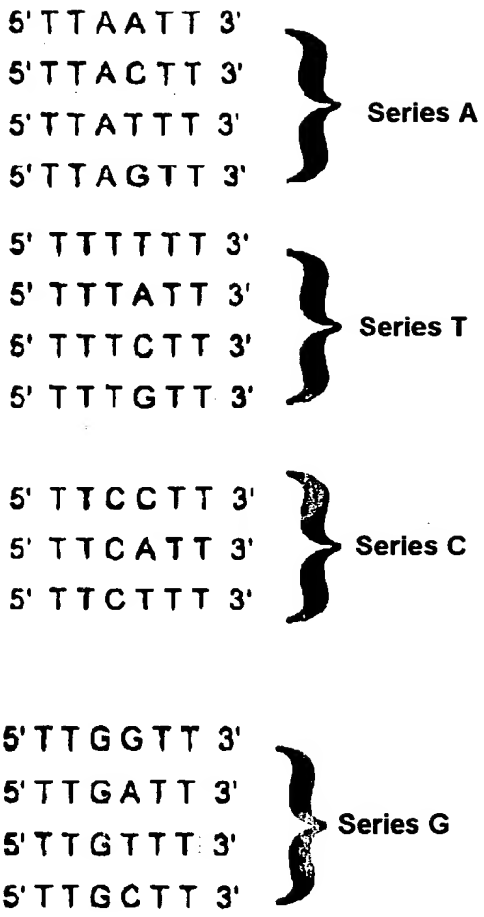
The immunization composition according to the invention may be intended for prophylactic administration or for therapeutic administration.

The immunization composition according to the invention may be formulated so as to optimize the adjuvant action of the oligonucleotide which is the subject of the invention. Thus, the oligonucleotide may be coupled to a lipid, such as cholesterol; it may be integrated into an emulsion of the oil/water type or formulated in the form of liposomes.

The following examples illustrate particular embodiments of the present invention.

Example 1: Oligonucleotide synthesis

15 oligonucleotides were synthesized, each having one of the following units:






and having 4 adenines in 5′ and 5 adenines in 3′.

These oligonucleotides are synthesized using an automatic synthesizer supplied by Applied Biosystems, which uses the standard phosphoramidite chemical method and which comprises, in each cycle, an oxidation step, which is carried out using a tetraethylthiuram/acetonitrile solution, in order to obtain a phosphorothioate bond.

An oligonucleotide A15(S) which comprises only As and which is phosphorothioate throughout its length is also prepared, in the same way. This oligonucleotide is a negative control since it causes neither proliferation nor an increase in the expression of activation markers on B lymphocytes.

An oligonucleotide 3Db(S), the sequence of which is identified in patent application WO96/02555 under SEQ ID No. 15 (5'GAGAACGCTCGACCTTCGAT3'), is also prepared; this oligonucleotide comprises phosphorothioate bonds throughout its length and is used as a positive control.

All the oligonucleotides are maintained in solution in PBS buffer.

Example 2: Lymphoproliferation test

Lymphocytes are isolated from the peripheral blood of a donor by carrying out a centrifugation on a Ficoll gradient. These lymphocytes are adjusted to $2\times10^6$ cells/ml in culture medium (RPMI 1640 + 10% fetal calf serum, and also glutamine, streptomycin and penicillin).

The cells are distributed into 96-well plates (round-bottomed) in 100 µl, i.e. $2\times10^5$ cells per well. 100 µl of a 4 µM solution of oligonucleotides to be tested produced in Example 1 (a single type of oligonucleotide per well) are then added in order to obtain a 2 µM final concentration.

The cells are incubated for 48 to 72 hours.

Tritiated thymidine (Amersham TRK 120) is diluted in culture medium and then distributed in the plates in the proportion of 1 µCi per well in 50 µl. After incubation for 7 to 8 hours in an incubator (5% $CO_2$, 37°C), the plates can be frozen at -80°C and treated later.

Using a "Harvester", the content of the wells is harvested onto Unifilter GF/C plates and 6 washes in distilled water are carried out followed by a wash in 70% ethanol in order to precipitate the DNA.

After drying the plates, 25 µl of liquid scintillant (Microscint-40, Packard) are distributed into each well and allow the radioactivity (radiation emitted by tritium) to be quantified by measuring the number of counts/minute (cpm) emitted by each well on a Top Count counter (Packard).

The results obtained for each of the oligonucleotides tested are represented on figures 1 and 2, which indicate, for each oligonucleotide tested, the number of counts per minute; it is noted that all the nucleotides according to the invention have a result which is clearly greater than the result obtained with the medium alone or the negative control A15(S), which means that they are all capable of stimulating lymphocyte proliferation.

Example 3:

Test relating to activation markers

The test is carried out using lymphocytes isolated from a donor as described in the previous example, and adjusted to $2\times10^6$ cells/ml in the same culture medium.

The cells are then distributed into 12-well plates in a volume of 2 ml, i.e. $4\times10^6$ cells/well. An amount of oligonucleotides to be tested prepared in Example 1 (1 oligonucleotide/well) which is sufficient to obtain a 2 µM oligonucleotide concentration is added to each well. The cells are then incubated for 72 hours at 37°C. The cells are then double-labeled using CD25PE/CD20FITC or CD86PE/CD20FITC, followed by analysis on a FACScan. The results obtained are illustrated on figures 3, 4, 5 and 6, which represent, for each oligonucleotide tested, the percentage of B cells (CD20+) which express the CD25 receptor (those which are CD25+) or the CD86 marker (those which are

CD86+). The results represented on figures 3 and 4 were obtained in a test carried out at a different time from the test for which the results are illustrated on figures 5 and 6, which explains the difference in the order of magnitude of the results obtained. Specifically, in this type of manipulation, the tests are very variable from one assay to the other, and only the results obtained in the same test are comparable with one another, hence the necessity of including, in each test, an oligonucleotide-control and also an assay of the medium alone.

It is noted that all the oligonucleotides which are subjects of the invention activate the B lymphocytes which express their activation marker CD86, and also the cytokine receptor CD25.

Example 4:

In the same way as in Example 1, a series of 16 oligonucleotides are prepared, which have the following sequences:

Seq ID 1 : 5′ TTAGTTATTAGTTATTAGTT 3′
Seq ID 2 : 5′ TTAGTTATTAGTTTTTAGTT 3′
Seq ID 3 : 5′ TTAGTTATTAGTTCTTAGTT 3′
Seq ID 4 : 5′ TTAGTTATTAGTTGTTAGTT 3′
Seq ID 5 : 5′ TTAGTTTTTAGTTATTAGTT 3′
Seq ID 6 : 5′ TTAGTTTTTAGTTTTTAGTT 3′
Seq ID 7 : 5′ TTAGTTTTTAGTTCTTAGTT 3′
Seq ID 8 : 5′ TTAGTTTTTAGTTGTTAGTT 3′
Seq ID 9 : 5′ TTAGTTCTTAGTTATTAGTT 3′
Seq ID 10 : 5′ TTAGTTCTTAGTTTTTAGTT 3′
Seq ID 11 : 5′ TTAGTTCTTAGTTCTTAGTT 3′
Seq ID 12 : 5′ TTAGTTCTTAGTTGTTAGTT 3′
Seq ID 13 : 5′ TTAGTTGTTAGTTATTAGTT 3′
Seq ID 14 : 5′ TTAGTTGTTAGTTTTTAGTT 3′
Seq ID 15 : 5′ TTAGTTGTTAGTTCTTAGTT 3′
Seq ID 16 : 5′ TTAGTTGTTAGTTGTTAGTT 3′

These oligonucleotides are of the phosphorothioate type throughout their length.

Example 5:

The capacity of the oligonucleotides prepared in Example 4 to induce human lymphocyte proliferation is evaluated using a lymphoproliferation test such as the one described in Example 2. In the same way as in Example 2, the oligonucleotide concentration per well is 2 μM and the controls consist of the medium alone, the oligonucleotide A15(S) and the oligonucleotide 3Db(S).

The results obtained, expressed in counts per minute, are represented in figure 7, which shows that all the oligonucleotides according to the invention are capable of inducing lymphocyte proliferation and that particularly good results are obtained when the sequences of the oligonucleotides are those identified by Seq IDs 9 to 12, i.e. when cytosine separates the first two $TTN_1N_2TT$ units of the oligonucleotide.

Example 6:

The capacity of the oligonucleotides prepared in Example 4 to induce the expression of the activation marker CD86 and of the receptor CD25 on B lymphocytes is evaluated. This evaluation is carried out using the test described in Example 3. The results obtained with the oligonucleotides prepared according to Example 4 are represented on figures 8 and 9, which illustrate the percentages of B cells (CD20+) which also express the receptor CD25 (figure 8) or the marker CD86 (figure 9).

The results obtained in this test confirm those obtained in the lymphoproliferation test: all the

oligonucleotides according to the invention induce the expression of activation markers on human B lymphocytes; particularly good results are obtained when the first 2 $TTN_1N_2TT$ units of the oligonucleotide are separated by cytosine.

Example 7:

The capacity of the oligonucleotides according to the present invention to induce cytokine secretion is evaluated.

For this evaluation, lymphocytes are isolated from the peripheral blood of a donor by carrying out a centrifugation on a Ficoll gradient. These lymphocytes are adjusted to $2\times10^6$ cells/ml in culture medium (AIM V medium + streptomycin + penicillin).

ELISPOT 96-well plates (flat bottom made of nitrocellulose) are pre-incubated the day before with a solution of antibodies for capturing cytokines (IL-10 or γ IFN depending on the test carried out), and then saturated with culture medium.

Next, 100 µl of cells are distributed into the ELISPOT plates, i.e. $2\times10^5$ cells per well, and then 100 µl of a 4 µM solution of oligonucleotides to be tested, produced according to Example 4 (a single type of oligonucleotide per well) are added in order to obtain a 2 µM final concentration. The test is carried out with the oligonucleotides having the sequences described under Seq ID 9, Seq ID 10, Seq ID 11 and Seq ID 12.

The plates are incubated at 37°C, under a 5% $CO_2$ atmosphere. After incubation for 72 hours, the cells are removed by washing in the presence of detergent (1% Tween) and the cytokines attached to the capture antibodies are revealed by successively adding

biotinylated detection antibodies (anti-IL-10 or anti-γ-IFN depending on the test carried out), streptavidin-HRP and the AEC substrate.

The spots (1 spot corresponding to 1 cytokine-secreting cell) are counted using an automatic counter. The results are expressed as number of spots (number of secretor cells) per million cells.

The results obtained for each of the oligonucleotides tested are represented on figures 10 and 11, which indicate, for each oligonucleotide tested, the number of cytokine-secreting cells per million total cells; it is noted that all the oligonucleotides according to the invention have a result which is clearly greater than the result obtained with the medium alone or the negative control A15(S), which means that they are all capable of inducing cytokine secretion, in particular IL10 and γ interferon secretion.